INCORPORATION OF AMINO ACIDS INTO PROTEIN BY ARTIFICIALLY ACTIVATED NON-NUCLEATE FRAGMENTS OF SEA URCHIN EGGS

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Early experiments by Hultin (1950, 1952) and by Hoberman, Metz and Graff (1952) showed that fertilization results in a considerable increase in the incorporation of labeled constituents into protein by sea urchin eggs. That the increase was not due to an increase in permeability was shown by experiments (Nakano and Monroy, 1958) on preloading the eggs, before ovulation, with labeled amino acid and by later experiments (Hultin and Bergstrand, 1960) on cell-free amino acid incorporating systems. Tests (Hultin, 1961) in which there was exchange of ribosomes and supernatants of such systems prepared from fertilized and unfertilized eggs indicated that ribosomal inactivity was responsible for the low capacity of the unfertilized egg to incorporate amino acid into protein, a view that receives additional support from the finding (Monroy, Maggio and Rinaldi, 1965) that trypsin treatment can "activate" the ribosomes of unfertilized eggs. On the other hand, it has been shown (Tyler, 1962, 1963; Nemer, 1962; Nemer and Bard, 1963; Wilt and Hultin, 1962) that ribosomes from unfertilized eggs respond to the synthetic messenger RNA polyuridylic acid, and do so as effectively as do those from the fertilized eggs or later embryos. This could be interpreted to mean that the relative inertness of the unfertilized egg was due to a lack of messenger RNA which was then formed upon fertilization, presumably as a result of some stimulus to the zygote nucleus. For further exploration of this proposition non-nucleate fragments of sea urchin eggs were prepared and measurements made, on homogenates as well as on the intact cells, of the effect of parthenogenetic activation on their ability to incorporate amino acids into protein (Tyler, 1963; Denny and Tyler, 1964). This was found to be similar to that of the nucleate fragment or the fertilized whole egg. Independent autoradiographic studies of incorporation by artificially activated intact fragments (Brachet, Ficq and Tencer, 1963) gave similar results. Other experiments (Gross and Cousineau, 1963; Gross, 1964) showed that dactinomycin (actinomycin D), which inhibits DNA-primed RNA synthesis, fails to suppress the fertilization-induced activation of incorporation of amino acid into protein and does not block early development. Further it has been found (Maggio, Vittorelli, Rinaldi and Monroy, 1964) that RNA extracted from unfertilized eggs can act *in vitro* as a template for protein synthesis.

From these experiments it has been concluded that the mRNA for the protein synthesis of early development is present in the ripe unfertilized egg but in an inactive, or "masked," form. There is, however, also evidence (Nemer, 1963; Wilt, 1963, 1964; Glišin and Glišin, 1964; Gross, Malkin and Moyer, 1964; Gross,

Kraemer and Malkin, 1965; Baltus, Quertier, Ficq and Brachet, 1965; Comb, Katz, Branda and Pinzino, 1965; Spirin and Nemer, 1965) that new RNA is synthesized during early development and that some of this is mRNA. It has been suggested on the basis of several lines of evidence (*cf.* Tyler, 1963, 1965) that this new mRNA, in turn, remains in a masked form, which may be represented by particles that have been termed informosomes (Spirin, Belitsina and Aitkhozhin, 1964; Spirin and Nemer, 1965), until a later stage of development. Further support of this view has been provided by electrophoretic studies (Spiegel, Ozaki and Tyler, 1965; Terman and Gross, 1966) showing that there are no marked qualitative differences in the pattern of soluble proteins that are formed during early development of sea urchin eggs and that development in the presence of dactinomycin did not detectably alter this pattern.

For further exploration of this problem the experiments with non-nucleate egg fragments have been extended to all 20 of the "coded" amino acids. In previous tests (Denny and Tyler, 1964; Baltus *et al.*, 1965) two different amino acids had been employed and the incorporation of each was stimulated by artificial activation of the fragment to an extent similar to that in the activated nucleated fragment or whole egg. The present experiments to which reference has been made earlier (Tyler, 1965) show this to hold for each of the 20 amino acids.

Materials and Methods

Eggs of the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* were obtained by KCl-injection of the animals and handled in artificial sea water (Tyler and Tyler, 1966). The separation into non-nucleate and nucleate fragments was done by the centrifugation method on layers of sucrose-sea water of graded density, essentially as described by Harvey (1956). Eggs of different species may differ in density and other properties, as may also eggs of the same species at different times and different conditions of maintenance of the animals. The optimum conditions for producing non-nucleate fragments may change accordingly (*cf.* Costello, 1938). The following procedure was most often effective for the eggs of the two species that were used. Isotonic sucrose (1.1 molar) solution and sea water were mixed in various proportions and layered in 30-ml. plastic centrifuge tubes. For *L. pictus* eggs the percentage of sucrose solution, and the volume, of each layer, starting from the bottom, was 75% (5 ml.), 52% (5 ml.), 50% (5 ml.) 45% containing the eggs (15 ml.). Centrifugation was for 15 minutes at 10,000 rpm in the SW 25.1 rotor of the Spinco model L centrifuge. Generally the non-nucleate fragments banded sharply above the 75% layer while the unsplit eggs were in, or on, the 50% layer, and the nucleate fragments near the top of the 45% layer. However, the positions varied to some extent, evidently partly because of variation in the time after preparation at which the tubes were used. *S. purpuratus* eggs are denser and require stronger force for separation into the fragments. For these the sucrose-sea water layers were generally 80% (3 ml.), 75% (4 ml.), $66\frac{2}{3}$% (10 ml.), 60% (10 ml.) and 50% containing the eggs (3 ml.). Centrifugation was for 5 minutes at 2500 rpm followed by 15 minutes at 10,000 rpm. The initial period at low speed served to stratify egg components so that the subsequent period of higher speed resulted in a more equal splitting into two fragments than if the initial period were omitted.

In most of the present experiments only the non-nucleate fragments of the centrifuged eggs were collected. This was usually done by puncturing the bottom of the centrifuge tube. Samples were examined to ascertain the absence of the nucleus and to measure the diameters. The fragments were generally uniform in size and had about half of the volume of the whole egg (average diameter = 112 μ for *L. pictus* and 78.4 μ for *S. purpuratus*).

For measurement of the incorporation of C^{14}-amino acid into protein the procedure of Mans and Novelli (1961), of plating the incubation mixtures on filter papers, was followed with some modifications (*cf.* Tyler, 1963; Tyler and Tyler, 1966). The filter papers ($1\frac{1}{2}'' \times 3''$, Whatman #1) were held with an intervening fold of aluminum foil at one end, in plastic clothespins mounted on a rack that supported also a manifold from which an airstream was directed to each paper. The quenched reaction mixtures (usually 0.30 ml.) were thus dried quickly after transfer to the papers, which were then dropped individually into a beaker of ice-cold 5% trichloroacetic acid (TCA). Generally not more than 2 dozen papers were processed together. However, larger numbers could be run in a single beaker without indication of transfer of radioactivity (tested by blank papers and by the t_o's). Further processing was with hot (90° C.) TCA, cool (20° C.) TCA, 95% ethanol, 100% ethanol, and ether, after which the papers (in the form of cylinders with non-overlapping edges) were transferred to the vials for determination of radioactivity by scintillation counting (Packard Tri-Carb Spectrometer).

The use of ice-cold TCA for the first treatment of the paper serves to reduce the amount of binding to the filter paper that occurs to various extent with the different amino acids, especially at elevated temperatures. Since in the present experiments whole eggs and intact fragments, rather than homogenates, were used these could be washed with sea water, after quenching with the C^{12}-amino acid, before transfer to the filter papers. This further reduced the opportunity for binding of the free C^{14}-amino acids to the filter paper. The values for the radioactivity of the t_o's (samples quenched with the C^{12}-amino acid solution at zero incubation time) in these experiments, were all very low and did not introduce a significant source of variation. The t_o's were mostly in the range of 0 to 60 cpm above background, and did not exceed 100 cpm in any of the tests except those with cystine, tryptophan and tyrosine, for which the values were in the range 50 to 150, 60 to 530 and 80 to 590, respectively.

For artificial activation of the non-nucleate fragments, and of the whole eggs, the single treatment with butyric acid was employed. An exposure of $1\frac{3}{4}$ minutes to a 0.005 molar solution proved to be optimum for both species. The procedure was to mix a small volume (usually 2 ml.) of the suspension with an equal volume of 0.01 molar butyric acid in a larger beaker and then add a large volume (usually 500 ml.) of sea water at the end of the treatment period.

Samples of the treated whole eggs and non-nucleate fragments were examined microscopically for determination of the per cent that were activated. As criteria of activation both membrane elevation and formation of the hyaline layer were used. The latter is particularly important for *L. pictus* since in this species the non-nucleate fragment may often fail to elevate a membrane on activation. Evidently in this species the separation of the fragments upon centrifugation causes a change (perhaps unrepaired rupture or loss) in the vitelline membrane such that there is

no membrane elevation upon activation in most cases. Thus membrane elevation for the fragments of *L. pictus* eggs ranged around 15% to 25% in the present experiments. However, over-all activation was above 90% for both the fragments and the whole eggs in all the experiments reported here for both species. Since the errors in scoring are generally in the direction of counting activated eggs as unactivated, it may be assumed that activation was close to 100% in all experiments. In any case a scoring error of some 10% is less than many of the differences between duplicate samples that derive from sampling error and other sources.

At one-half hour after the butyric acid treatment, in the principal experiments, 0.25-ml. aliquots of the washed, and concentrated (generally about 10,000 to 20,000 per ml.), suspensions of the whole eggs and non-nucleate fragments were distributed (calibrated, wide-mouth, droppers) in duplicate to plastic tubes (15 mm. diameter) containing 0.05 ml. of the C^{14}-amino acid in sea water, and incubated at 20° C. with gentle shaking for one hour. The zero time (t_0) blanks for each C^{14}-amino acid were also set up in duplicate with a large excess of the corresponding C^{12}-amino acid present and kept in an ice bath for processing with the others. The t_0's received aliquots of the whole eggs.

Determinations of the quantity of egg material used in an experiment were done by counting the eggs in samples of the suspensions (*cf.* Tyler and Tyler, 1966) and by centrifugation in a Hopkins vaccine tube at 2500 *g* for 10 minutes. The average values obtained for the packed cell volumes for 10^4 eggs, were 0.0029 cc. for *S. purpuratus* and 0.0083 cc. for *L. pictus*. The volumes occupied by 10^4 eggs of these two species as calculated from their average diameters are 0.0025 cc. and 0.0075 cc.

Experiments

Pattern of incorporation

Data for the incorporation of the 20 C^{14}-amino acids by activated whole eggs and activated non-nucleate fragments, in six experiments with *S. purpuratus* and six with *L. pictus*, are presented in Figures 1 and 2, respectively. In each experiment 10 amino acids were tested at one time with the whole eggs and non-nucleate fragments of the same batch of eggs. Comparisons may thus be made within each experiment, between whole eggs and non-nucleate fragments, on the basis of the pattern of incorporation of the 10 amino acids, apart from the absolute values, which involve the additional variation connected with determinations of egg quantity. Furthermore, in addition to its dependence on the kind of protein synthesized, the absolute value for incorporation of a particular C^{14}-amino acid also depends on the size of the intracellular pool of the corresponding C^{12}-amino acid, and this may vary from one batch of eggs to another.

In the data presented in Figures 1 and 2 the average values for the 10 different amino acids of an experiment are connected by broken lines. This was done so as to facilitate visualization of the pattern, the lines having no graphical significance. As the figures show, the patterns for the whole eggs and for the non-nucleate fragments are very much alike. Within each experiment whole eggs and non-nucleate fragments also show generally similar absolute values for incorporation of the individual amino acids. Occasional divergences that may be seen in one experiment are not observed in the other two of the same series. In certain of the

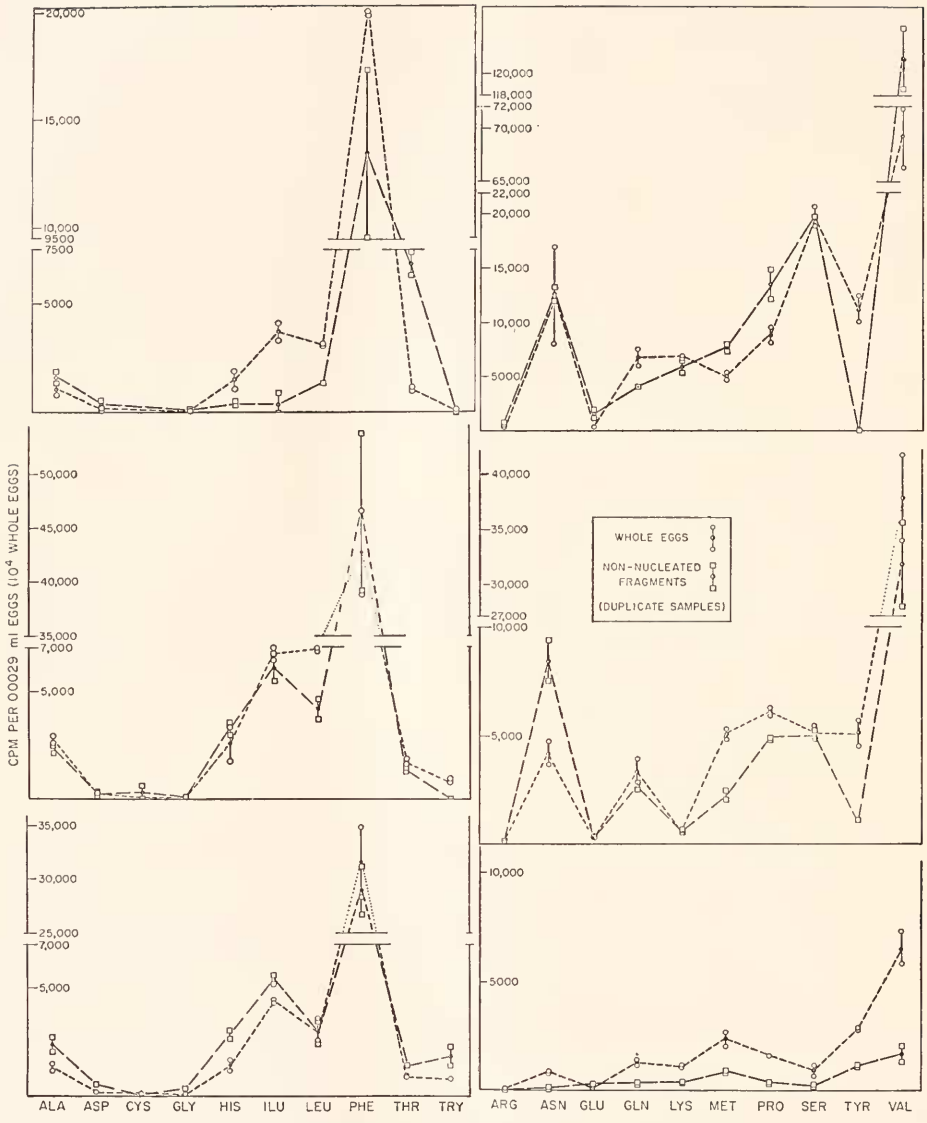

FIGURE 1. Incorporation of C^{14}-labeled amino acids into protein during one hour by non-nucleate fragments and whole eggs of *Strongylocentrotus purpuratus* at $\frac{1}{2}$ hour after activation with butyric acid. The individual amino acids were tested in groups of 10 in each experiment in duplicate samples of whole eggs and of non-nucleate fragments, and at 0.4 μc./ml. Three experiments with one group are presented in the three charts on the left and three with the other group on the right. Specific activities in cpm and names for abbreviations are: 4.24 (Alanine), 22.5 (Aspartic Acid), 7.70 (Cystine), 5.25 (Glycine), 22 (Histidine), 6.16 (Isoleucine), 6.3 (Leucine), 360 (Phenylalanine), 5.15 (Threonine), 8.95 (Tryptophan), 9.23 (Arginine), 22.5 (Asparagine), 205 (Glutamic Acid), 4.05 (Glutamine), 7.1 (Lysine, except 43.8 in upper right chart), 15.6 (Methionine), 7.1 (Proline), 1.7 (Serine), 11.54 (Tyrosine) and 200 (Valine). For the purpose of enabling the pattern of incorporation to be more readily visualized the averages are connected by dashed lines. The lines have no other significance.

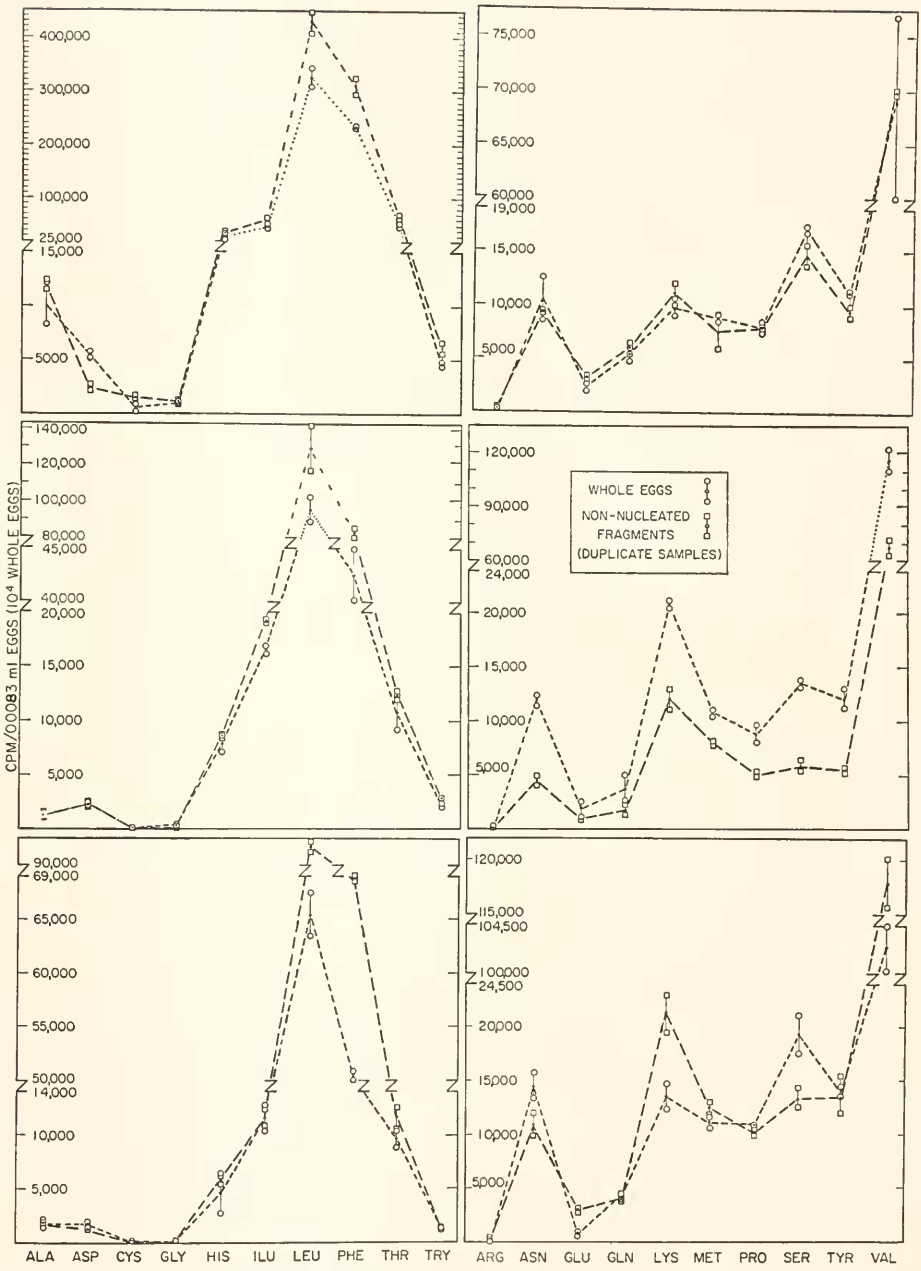

FIGURE 2. Same as Figure 1 but for eggs of *Lytechinus pictus* and with following differences in specific activities: 160 (Leucine), 25.6 (Threonine) and 43.8 (Lysine).

experiments, *e.g.* the middle one on the right of Figure 2, the absolute values for each of the 10 amino acids are lower in the non-nucleate fragments than in the whole eggs. This doesn't hold for the other two experiments with this set of amino acids, nor with the three involving the same set of amino acids in the other species. It does not, then, indicate a significantly lower capacity of the non-nucleate fragment for

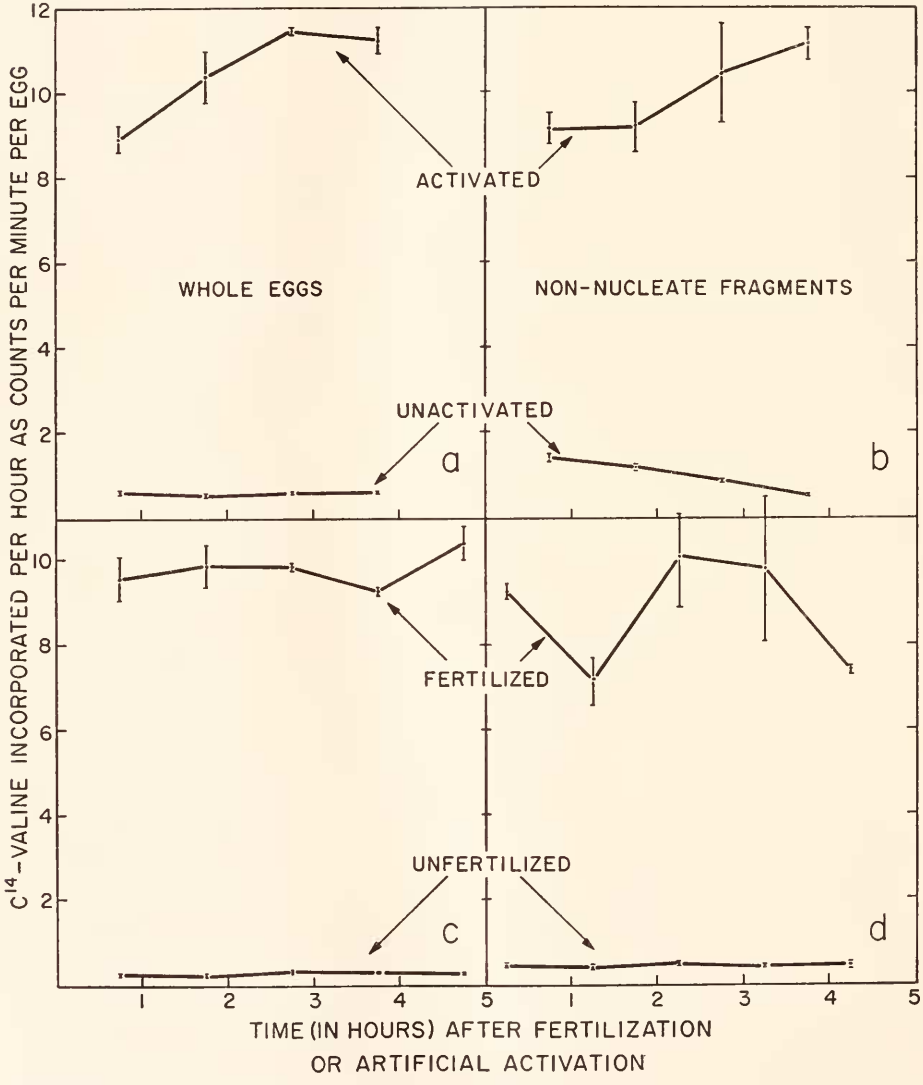

FIGURE 3. C^{14}-valine incorporated into protein in one hour by eggs of *Lytechinus pictus* starting at the indicated times. *a* and *b*, whole eggs and non-nucleate fragments of the same experiment. *c* and *d*, two separate experiments. Incubations at 20° C. in 0.3 ml. of C^{14}-valine (sp. act. 200 cpm) in sea water at 0.4 μc./ml. and at egg concentrations near 1% (3600 eggs) by volume. Activation with butyric acid as described. Vertical lines show spread of duplicates.

incorporation of the amino acids into protein. Most probably, in this experiment, the errors in estimation of egg quantity and in percentage activation were relatively greater than other sources of variation.

If the data are normalized with respect to any one amino acid, and account is taken of the fact that specific radioactivities differed in certain cases, it appears that the two species differ somewhat in pattern of incorporation. The non-nucleate fragments are similar to the whole eggs in this respect and also with respect to the absolute values for each amino acid.

Time course of incorporation

The incorporation of C^{14}-valine into protein by non-nucleate fragments and whole eggs of *Lytechinus pictus* was measured at various times during the first five hours after artificial activation. Data of one experiment are presented in Figure 3a and b, which includes also the unactivated controls. Data for two separate experiments with fertilized eggs, and their unactivated controls, are shown in Figure 3c and d.

From the results of this and another similar experiment it appears that the incorporation of C^{14}-valine by the activated non-nucleate fragment remains as high as that of the activated whole egg during this period of time. The small rise that appears in the data of Figure 3a and b is probably of no significance since it is not evident in the other experiment. The same may be said for the decrease with time shown by the control, unactivated, non-nucleate fragments.

Figure 3 also shows an approximate constancy of incorporation of the C^{14}-valine into protein on the part of the fertilized eggs during this period. The initial great increase in incorporation into protein occurs very soon after fertilization and no significant further increase appears during the first five hours.

In the experiment plotted in Figure 3c the eggs were distributed to the incubation tubes at one time and the C^{14}-valine added at the indicated time. In that of Figure 3d the eggs were distributed at the indicated times to the tubes. This difference in procedure probably accounts for the larger fluctuations obtained in the latter experiment.

Discussion

The present experiments show that the previously reported similarity of artificially activated non-nucleate fragments to artificially activated (or fertilized) whole eggs with respect to the incorporation of amino acid into protein, applies to all 20 of the coded amino acids. In individual experiments in which sets of 10 amino acids were tested at the same time the relative incorporation values for the non-nucleate fragments exhibit a pattern very similar to that of the whole eggs. Also the absolute values are similar. The use of two different species of sea urchins, differing somewhat in pattern of incorporation and absolute values, facilitated the comparisons. Further, in experiments in which incorporation of one of the amino acids was followed for several hours the non-nucleate fragment remained as active as the whole egg.

It may be concluded that the kinds of protein synthesized by the activated non-nucleate fragment do not differ appreciably from those of the whole egg during early

development. The previous experiments with non-nucleate fragments, the experiments with dactinomycin, and the demonstration of template activity of RNA extracts from unfertilized eggs, to which reference was made in the introduction, provided evidence that the cytoplasm of the unfertilized egg contains masked messenger (m)RNA that becomes active upon fertilization. The present experiments indicate also that it is this mRNA that serves for the bulk of protein synthesis in early development.

The data of Figure 3 show that, following the great increase shortly after fertilization, there is relatively little change in rate of incorporation of amino acid into protein during the first few hours of development. The occurrence, during early development, of a plateau in the rate of incorporation of amino acid into protein by sea urchin embryos has been reported by several other investigators (*e.g.,* Hultin, 1952, 1961; Markman, 1961; Giudice, Vittorelli and Monroy, 1962; Gross and Cousineau, 1964; Berg, 1965; *cf.* Monroy, 1965), although the reports differ to some extent in the time at which this occurs. One may infer that the mRNA molecules are all, or mostly all unmasked at the time that the plateau occurs. From the present data it appears that the complete unmasking occurs very soon after fertilization. These inferences presuppose that the mRNA remains stable during early development, an assumption that seems to accord with the results of experiments on the mRNA of other animal cells (*cf.* Marks, Burka, Conconi, Perl and Rifkind, 1965).

One may also infer from these results that if any significant amount of new mRNA is formed in early development it must remain largely inactive during this period. As stated in the introduction, convincing evidence has, in fact, been accumulating that new mRNA is produced during early cleavage stages of sea urchins. It was also surmised that this mRNA for the most part, remains masked until a later stage of development. There is now evidence (Spirin and Nemer, 1965) that the mRNA synthesized during early development of sea urchins occurs in the form of RNP particles, that are lighter than the ribosomes and also in light polyribosomes, neither of which are active in the incorporation of amino acid into protein. The latter activity is exhibited mainly by the heavy polyribosomes which do not become labeled by an RNA precursor administered during early development.

Reference was also made in the introduction to two electrophoretic studies (Spiegel *et al.,* 1965; Terman and Gross, 1966) showing no marked qualitative differences in the soluble proteins that are formed during early development. In the study by Terman and Gross (1966), in addition to the similarity in radioactivity pattern for newly synthesized proteins in control and actinomycin-treated embryos during the period of cleavage, systematic changes were also noted. These appeared in the autoradiograms from blastula extracts and became more obvious at gastrulation. Terman and Gross interpret this to imply either differences in the kinds of synthesized proteins or changes in rates of synthesis of species in the same set. Dactinomycin-treated embryos follow the controls with regard to these changes, which cannot, then, be attributed to the new mRNA that is synthesized during early development. Possibly, then, while the major portion of the masked mRNA present in the unfertilized egg is activated shortly after fertilization, there may be some selective activation of some masked messengers at later stages. In an initial investigation (Spiegel and Tyler, 1966) of this proposition, with micromeres, pre-

pared from sea urchin eggs at the 16-cell stage, incorporation of amino acid into protein was found to be quantitatively similar to that of the other cells.

Apart from the question of selective activation of various kinds of masked mRNA it appears that the production of mRNA in an initially inactive form occurs commonly, or perhaps invariably, during development. It has been previously indicated (Tyler, 1965), on the basis of a survey of experiments on the time of occurrence of dactinomycin sensitivity in the differentiation of a particular tissue, that the production of masked mRNA's represents the "determination process" in embryonic development. Actual differentiation, then, follows a later unmasking process which very likely acts at one time, or within a short period of time, on all the species of mRNA molecules involved in the differentiation of the tissue.

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Summary

1. Artificially activated non-nucleate fragments and whole eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* have been compared with regard to their ability to incorporate C^{14}-labeled amino acid into protein. Each of the 20 "coded" amino acids has been tested, the tests being done in groups of ten. In both species non-nucleate fragments closely resemble whole eggs with regard to the pattern of incorporation of the sets of ten amino acids. Also there is similarity in the absolute incorporation values when the comparisons are made within individual experiments in which variation in size of the endogenous amino acid pool is not a factor.

2. The similarity in amino acid (valine) incorporation is maintained during the first six hours after activation. The values are similar to those for fertilized eggs, as earlier studies had also shown. Further, following the initial great rise shortly after fertilization, or artificial activation, the rate of incorporation remains relatively constant during this period.

3. The results provide additional evidence that the activation of protein synthesis upon fertilization does not depend upon production of mRNA by the nucleus. Further they show that the activated non-nucleate fragment and the whole egg do not differ appreciably in the kinds of proteins they synthesize during early development, that the corresponding mRNA's are all, or mostly all present in the unfertilized egg, and that most or all of these become "unmasked" soon after fertilization or artificial activation.

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